532 Rec'd PCT/PTC 19 JUL 2000

FORM PTO-1390 (REV 11-98)	U S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 3673-3
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/600594 (To be assigned)
INTERNATIONAL APPLICATION NO. **PCT/BR99/00096**	INTERNATIONAL FILING DATE **23 November 1999**	PRIORITY DATE CLAIMED **23 November 1998**

TITLE OF INVENTION
MONITORING PATIENT COMPLIANCE AND BIOAVAILABILITY OF DRUGS BY DEPOTEINIZING BODY FLUIDS

APPLICANT(S) FOR DO/EO/US
FERREIRA et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has **NOT** expired.
   d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. To 16. Below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information. **International Search Report/ PTO-1449/ Two References**

532 Rec'd PCT/PTO 19 JUL 2000

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/600594 (to be assigned)	INTERNATIONAL APPLICATION NO. **PCT/BR99/00096**	ATTORNEY'S DOCKET NUMBER **3673-3**

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5):

-- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $970.00

-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $840.00

-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2) paid to USPTO $690.00

-- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) $670.00

-- International preliminary examination fee paid to USPTO (37 C.F.R. 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $96.00

						CALCULATIONS		PTO USE ONLY
ENTER APPROPRIATE BASIC FEE AMOUNT =						$	970.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).						$	130.00	
CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE				
Total Claims	21	-20 =	1	X	$18.00	$	18.00	
Independent Claims	3	-3 =	0	X	$78.00		0.00	
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)				$260.00		$	0.00	
TOTAL OF ABOVE CALCULATIONS =						$	**1118.00**	
Reduction by ½ for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 C.F.R. 1.9, 1.27, 1.28).							0.00	
SUBTOTAL =						$	**1118.00**	
Processing fee of $130.00, for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)). +							0.00	
TOTAL NATIONAL FEE =						$	**1118.00**	
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28. 3.31). **$40.00** per property +						$	0.00	
Fee for Petition to Revive Unintentionally Abandoned Application ($1210.00 – Small Entity = $605.00)						$	0.00	
TOTAL FEES ENCLOSED =						$	**1118.00**	
						Amount to be: refunded	$	
						Charged	$	

a. ☒ A check in the amount of $1118.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 14-1140 in the amount of $_____ to cover the above fees. A duplicate copy of this form is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.

d. ☐ The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application.

NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

NIXON & VANDERHYE P.C.
1100 North Glebe Road, 8th Floor
Arlington, Virginia 22201
Telephone: (703) 816-4000

Michelle N. Lester (signature)
SIGNATURE

Michelle N. Lester
NAME

32,331 — **July 19, 2000**
REGISTRATION NUMBER — Date

PTO/PCT Rec'd 07 SEP 2000
09/600594

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

FERREIRA et al. Atty. Ref.: **3673-3**

Serial No. **09/600,594** Group: **unknown**

Filed: **July 19, 2000** Examiner:

For: **MONITORING PATIENT COMPLIANCE AND BIOAVAILABILITY OF DRUGS BY DEPOTEINIZING BODY FLUIDS**

* * * * * * * * * * *

PRELIMINARY AMENDMENT

September 7, 2000

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

It is respectfully requested that the following amendment be entered in the subject allowed application.

<u>IN THE SPECIFICATION:</u>

Page 1, after the title and before first line of text, insert as a separate paragraph:

--This application is the national phase of international application PCT/BR99/00096 filed November 23, 1999 which designated the U.S.--.

REMARKS

By this amendment, the specification has been amended to reference the PCT application of which this application is the U.S. National Phase, as requested by 37 CFR

§120. No new issues are raised by this amendment. Accordingly, early entry of this amendment is respectfully requested.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: [signature]

Michelle N. Lester
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09/600594
532 Rec'd PCT/PTO 19 JUL 2000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

FERREIRA et al. Atty. Ref.: **3673-3**

Serial No. **(To be assigned)** Group:

National Phase of **PCT/BR99/00096**

Filed: **July 19, 2000** Examiner:

For: **MONITORING PATIENT COMPLIANCE AND BIOAVAILABILITY OF DRUGS BY DEPOTEINIZING BODY FLUIDS**

* * * * * * * * * * *

July 19, 2000

Assistant Commissioner for Patents
Washington, DC 20231
Sir:

PRELIMINARY AMENDMENT

Prior to calculation of the filing fee and in order to place the above identified application in better condition for examination, please amend the claims as follows:

IN THE CLAIMS

Claim 4, line 1, delete "to 3",

Claim 9, line 1, change "claims 1, 3, 5 and 7" to --claim 1--,

Claim 14, line 1, change "claims 11 and 13" to --claim 11--.

REMARKS

The above amendments are made to place the claims in a more traditional format.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By [signature]
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OMB No. 0651-0011 (12/31/86) #4

Applicant or Patentee: **FERREIRA et al.** Attorney's Dkt. No. **3673-3**

Serial or Patent No.:

Filed or Issued: **July 19, 2000**

For: **MONITORING PATIENT COMPLIANCE AND BIOAVAILABILITY OF DRUGS BY DEPOTEINIZING BODY FLUIDS**

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS [37 19(f) and 1.27(c)] - SMALL BUSINESS CONCERN

I hereby declare that I am

- [ ] the owner of the small business concern identified below:
- [x] an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN FUNDACAO OSWALDO CRUZ – FIOCRUZ

ADDRESS OF CONCERN **Avenida Brasil 4365, 21045-900, Manguinhos, Rio de Janeiro RJ Brasil**

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled:

MONITORING PATIENT COMPLIANCE AND BIOAVAILABILITY OF DRUGS BY DEPOTEINIZING BODY FLUIDS

by inventors **FERREIRA et al.** described in

- [ ] the specification filed herewith.
- [x] application Serial No. 09/600,594, filed **July 19, 2000**
- [ ] patent No. , issued

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor who could not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 CFR 1.27)

Name

Address

- [ ] Individual - [ ] Small Business Concern - [ ] Nonprofit Organization

Name

Address

- [ ] Individual - [ ] Small Business Concern - [ ] Nonprofit Organization

I acknowledge the duty to file, in this application of patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28(b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Eloi de Souza Garcia

TITLE OF PERSON OTHER THAN OWNER President

ADDRESS OF PERSON SIGNING Av. Brasil 4365, 21045-900, Manguinhos, RJ/BR

SIGNATURE [signature] DATE 23 August 2000

Dr. ELOI DE SOUZA GARCIA
Presidente da FIOCRUZ

443933

8/PRTS

Title: MONITORING PATIENT COMPLIANCE AND BIOAVAILABILITY OF DRUGS BY DEPROTEINIZING BODY FLUIDS

The present invention relates to methods of determining the concentration of a selected drug in the body of a subject to provide the monitoring of either drug levels in a clinical setting and in public health services and patient compliance with medication prescriptions. The methods are characterized by a simplified and effective deproteinizing body fluid step followed by drug extraction and measurement using an accurate technique, such as a colorimetric assay or a High-Performance Liquid Chromatography method.

BACKGROUND OF THE INVENTION

In the field of medicine, a number of medications have been found safe and efficacious for the treatment of patients with physical illnesses. Patients placed on prescribed medication treatment plans are typically monitored. Subjective and objective methods are used to identify bothersome symptoms and to implement any changes necessary during the course of treatment. Monitoring may continue for as long as treatment is provided.

Currently, the most common method of monitoring patients for medication compliance is clinical observation which involves individual counseling and close personal supervision by physicians which observe physiological signs and symptoms or residual signs of illness and also listen to patient complaints regarding degree of pain relief and evaluate

physiological changes over time. This method is time consuming, expensive and highly subjective. Needless to say, it is fraught with potential errors.

Additional compliance information can also be obtained using qualitative urine monitoring methods such as the standard laboratory procedure called enzyme-multiplied immunoassay (EMIT). Utilizing an arbitrary cutoff value, these methods provide the clinician with a simple positive or negative indication of the possible presence or absence of a parent drug or its metabolites in a patient's urine. The parent drug is the prescribed medication itself and the metabolites are those chemical derivatives of the medication which naturally occur upon the patient's body metabolizing the medication. These tests do not provide information concerning the time or amount of last drug use or whether or not the prescribed dose of medication was ingested properly, diverted or supplemented.

Physicians utilizing only clinical evaluation and qualitative urine drug screening test results may develop problems in their treatment methods. Consistently, Fox, W. (Fox, W. (1990). "Drug combinations and bioavailability of rifampicin". Tubercle. 71: 241-5) suggested parallel serum/plasma sampling in selected studies for testing abroad to verify the tuberculosis treatment effectiveness using drug combinations by confirming the urinary bioavailability determination. In the mentioned text, the term "abroad" means

outside developing countries in which expensive analytical equipment is not commonly found.

Another monitoring method sometimes used is a direct measurement of parent drug concentrations or active metabolites concentrations of the drug in plasma and other body fluids. This direct method presents some limitations since it is expensive and requires the use of time consuming and highly technical analytical procedures such as high-performance liquid chromatography and mass spectrometry since active and inactive metabolites must be quantified separately.

Attempts have been made to overcome the difficulties of the sophisticated analytical procedures. In the EP 122 032, it is described a method of determining the concentration of a selected drug in the body of a subject consisting of the steps of holding a liquid collecting means comprising an absorbent inert member, containing a reagent substance which reacts with selected drug, in a position in close proximity to an eye of the patient for collecting tear fluid therefrom and allowing the tear fluid collected to come into contact with said reagent substance during a period sufficient to permit the development of the reaction which has to be physically detectable. It is mentioned that this method provides a readily indication of the level of said drug in the body because the tear fluid is less complex then other

body fluids such as blood. Nevertheless, this assay permits only qualitative or semi-quantitative drug detection.

Although simplicity is an important quality when dealing with monitoring methods, the accuracy of the assay is crucial in the control of diseases, e.g. tuberculosis, specially to measure small quantities of drugs in complex body fluids, such as blood. In the US 4 656 141 it is proposed a high-performance liquid chromatography process for detecting the presence of trace amounts of non-fluorescent soluble compounds each having at least one labile hydrogen atom in a carrier solution by adding a non-fluorescent quinone which is reducible to a fluorescent hydroquinone, and irradiating the resulting solution in the absence of oxygen with light of sufficient energy to cause the quinone to be reduced to a hydroquinone.

Preferably both quantitative and analytical methods should be used to follow the patient on a repetitive basis to ensure that the patient is indeed ingesting the prescribed amounts of medication in the proper manner and responding as expected. Moreover, in control programmes of Public Health Services, confident monitoring of treatment is crucial. Tuberculosis Control Program may be cited as a representative example of this approach and Rifampicin as a highly potent drug widely used for tuberculosis treatment.

An efficient follow up the other drugs treatment performance is also important. Examples are anti-retroviral

drugs, such as proteinase or reverse transcriptase inhibitors, e.g. 2′,3′-dideoxyinosine (ddI), 2′,3′-dideoxycytidine (ddC) or 3′-azido-2,3′-dideoxythymidine (AZT) (see Frijus-Plessen N., Michaelis H.C., Foth H. e Kahl G.F. (1990). "Determination of 3′-azido-3′-deoxythymidine, 2′,3′-dideoxycytidine, 3′-fluoro-3′-deoxythymidine and 2′,3′-dideoxyinosine in biological samples by high-performance liquid chromatography". Chrombio. Elsevier Science Publishers B.V. Amsterdam. 534: 101-107), anti-fungal drugs, e.g. itraconazole which is also used in anti-leishmanial chemotherapy (Anon: British Society for Antimicrobial Chemotherapy Working Party: Laboratory monitoring of antifungal chemotherapy. The Lancet. Vol. 337. pp. 1577-1580. 1991) or antimonials, the most used anti-leishmanial drug (World Health Organization. Tropical Disease Research. Twelfth Programme Report. World Health Organization. Geneva. Switzerland. Pp 139.1995).

In the case of patients with tuberculosis, there has been increasing interest in the determination of serum levels of the main antituberculosis drugs, in particular the most used rifampicin medication. The usual methods for rifampicin assay are colorimetry, microbiology and high-performance liquid chromatography. In the beginning, microbiological assays were employed by using *Sarcina lutea* or *Staphylococcus aureus*. Examples are described in: Furesz S., Scotti, R., Pallanza R., Mapelli E. (1967). "Rifampicin: A new

rifampicin. III Absorption, distribution and elimination in man". Arzneim-Forsch. 17: 534-7; Boman, G. (1974). "Serum concentration and half-life of rifampicin after simultaneous oral administration of aminosalicylic acid or isoniazid". Europ J Clin Pharmacol. 7: 217-25; Dickinson, J.M., Aber, V.R., Allen, B.W., Ellard, A., Mitchison, D.A. (1974). "Assay of rifampicin in serum". J Clin Path. 27: 457-62; Buniva, G., Pagani, V., Carozzi, A. (1983). "Bioavailability of rifampicin capsules". Int J Clin Pharmacol Therapy Toxicol. 21: 404-9; Immanuel, C., Jayasankar, K., Narayana, A.S.L., Saema, G.R. (1985). "Self-induction of rifampicin metabolism in man". Indian Med Res. 82: 381-7. However, the precision of such methods is generally poorer than would be expected with HPLC methods.

Colorimetric methods are interesting under the point of view of easier accomplishing. The procedures of such methods are described in: Maggi, N., Furesz, S., Pallanza, R., Pelizza G. (1969). "Rifampicin desacetylation in the human organism". Arzneim-Forsch. 19: 651-4; Sunahara, S., Nakagawa, H. (1972). "Metabolic study and controlled clinical trials of rifampicin". Chest. 61: 526-32; Jeanes, C.W.L., Jessamine, A.G., Eidus, L. (1972). "Treatment of chronic drug-resistant pulmonary tuberculosis with rifampicin and ethambutol". Canad Med Ass J. 106: 884-8; Brechbuhler, S., Flueher, H., Riess, W.(1978). "The renal elimination of rifampicin as a function of the oral dose". Arzneim-Forsch. 28: 480-3; McConnell,

J.B., Smith, H., Davis, M., Williams, R. (1979). "Plasma rifampicin assay for an improved solvent extraction technique". Br J Clin Pharmc. 8: 506-7; Israili, Z.H., Rogers C.M., El-Attar, H. (1987). "Pharmacokinetics of antituberculosis drugs in patients". J Clin Pharmacol. 27: 78-83

High-Performance Liquid Chromatography (HPLC) has been used for separate determination of rifampicin and its metabolites. HPLC procedures are described in: Goucher, C.R., Peters, J.H., Gordon, G.R., Murray, J.F., Ichikawa, W., Welch, T.M., Gelber, R.H. (1977). "Chemical and bacteriological assays of rifampicin, rifampicin-quinone and desacetylrifampicin". 12th U.S.-Japan Joint Conference on Leprosy. Boston. Ma. Sept 27-29, 1977. pp. 47-59; Lecaillon, J.B., Febvre, N., Metayer, J.P., Souppart, C.(1978). "Quantitative assay of rifampicin and three of its metabolites in human plasma, urine and saliva by high-performance liquid chromatography". J Chromatogr. 145: 319-24; Ratti, B., Rosina-Parenti, R., Toselli A., Zerrili, L.F. (1981). "Quantitative assay of rifampicin and its metabolite 25 desacetyl-rifampicin in human plasma by reversed-phase high-performance liquid chromatography". J Chromatogr. 225: 526-31; Guillaumant, M., Leclercq, M., Forbert, Y., Guise, B., Harf, R. (1982). "Determination of rifampicin, desacetylrifampicin, isoniazid and acetylisoniazid by high performance liquid chromatography: Application to human serum

extracts, polymorphonucleocytes and alveolar macrophages". J Chromatogr. 232: 369-76; Acocella, G., Nonis, A., Gialdroni-Grassi, G., Grassi, C. (1988). "Comparative bioavailability of isoniazid, rifampicin, and pyrazinamide administered in free combination and in a fixed triple formulation designed for daily use in antituberculosis chemotherapy". Am Rev Respir Dis. 138: 882-5; Ishii, M., Agata, H. (1988) "Determination of rifampicin and its main metabolites in human plasma". J Chromatogr. 426: 412-6; Nau, R., Prange, W.H., Menck, S., Kolenda, H., Visser, K., Seydel, J.K. (1992). "Penetration of rifampicin into the cerebrospinal fluid of adults with uninflamed meninges". J Antimicrob Chemother. 29: 719-24; Chouchane, N., Barre, J., Toumi, A., Tillement, J.P., Benakis, A. (1995). "Bioequivalence study of two pharmaceutical forms of rifampicin capsules in man". Eur J Drug Metab Pharmacokin. 20: 315-20.

While providing useful information relative to patient status and treatment compliance, the clinical monitoring methods described above, i.e. clinical interviews with patients, direct plasma drug measurement and qualitative urine drug screening, have distinct drawbacks which limit their usefulness in extended treatment programmes. Although being effective, the complex assays with many extraction steps, e.g. HPLC, require expensive equipment and specialized operating personel and materials which are not easily found in small hospital centers or field laboratories, mainly in

developing countries. Moreover, the occurence of losses during the extraction steps lead to lower drug concentrations, and consequently to wrong results.

Thus, it remains a need for methods of monitoring patient compliance whithout the above mentioned disadvantages of the known methods but having sensitivity and specificity sufficient to detect trace amounts of substances contained in complex body fluids. Such monitoring methods would help physicians both in prescribing adequate doses of medication and in monitoring patients to insure that they are ingesting the prescribed amounts. Accordingly, it is to the provision of such improved methods that the present invention is directed.

SUMMARY OF THE INVENTION

The object of the invention is to provide the monitoring of either drug levels in a clinical setting and in public health services and patient compliance with medication prescriptions. The drug levels monitoring is accomplished by quantitative assays which allow drug detection in body fluids down to 0.3 µg/ml. The method based on extraction of the drug from biological fluids is characterized by a prior deproteinizing step in conditions that at least 97% of the drug is recovered, i.e. by carrying out the deproteinizing step in the presence of $ZnSO_4$ it is possible to efficiently strip off the drug which became bound to proteins contained in the biological fluid. Noteworthy the method of the

invention is specially useful for a drug assay from blood which contains much more protein than other biological fluids such as urine, saliva, tear fluid.

One embodiment of the present invention is a method for drug level detection by using a simplified and effective deproteinizing step from body fluids, such as plasma, blood, urine, saliva, tear fluid, followed by drug extraction and measurement using an accurate technique, such as a colorimetric assay or a High-Performance Liquid Chromatography method.

In a particular embodiment, the invention is directed to a method to quantify rifampicin in order to monitor its levels in body fluids and also to a kit of tuberculosis diagnosis based on rifampicin concentration measurement.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows the reproducibility and accuracy of the method of the present invention illustrated by HPLC chromatograms of synthetic mixtures of rifampicin with body fluids: (A) rifampicin in plasma at 25; 12,5; 6,25; 3,13 and 1,56 µg/ml; (B) various samples of rifampicin synthetic mixture in saliva at a concentration of 2.0 µg/ml; (C) various samples of rifampicin synthetic mixture in urine at a concentration of 18 µg/ml.

FIGURE 2 shows the reproducibility and accuracy of the method of the present invention illustrated by a HPLC chromatogram of a synthetic mixture of 2',3'-dideoxycytidine

(ddC), 2',3'-dideoxyinosine (ddI) and 3'-azido-2,3'-dideoxythymidine (AZT) with plasma at a concentration of 20 µg/ml.

FIGURE 3 shows the reproducibility and accuracy of the method of the present invention illustrated by a HPLC chromatogram of a synthetic mixture of itraconazole with plasma at concentrations of 20; 10; 5; 2,5; and 1,25 µg/ml.

FIGURES 4A and 4B illustrate a set of standard curves demonstrating that Beer's law is followed for the range of 0.39 to 25 µg/ml of rifampicin in plasma, and the reproducibility of the method of the present invention by colorimetric measurements at 340 nm.

FIGURE 5 illustrates the usefulness of the method of the present invention in rifampicin pharmacokinetics studies, showing the variation of rifampicin plasma concentration at the indicated time intervals for two HIV positive patients with tuberculosis.

DETAILED DESCRIPTION OF THE INVENTION

In medication maintenance programs, the patient is initially prescribed by a medication and dose based on several factors. These ordinarily include the severity and duration of illness, amounts and types of medications previously used, previous medical history, patient sex, pregnancy status, patient weight and ingestion of other therapeutic medications. In certain instances, the pathogenic agent may develop a significant level of resistance to the

drug or therapeutic combinations and therefore a loss of sensitivity to the administered drug. In this respect, the regular intake of drugs is of great importance.

To determine compliance with the prescribed medication dose, random body fluid samples, e.g. urine or blood are obtained from the patient and parent drug and/or its metabolites concentration is measured. Consistently, antifungal drugs concentrations in blood are measured either to ensure adequate concentrations of the drug and to avoid unwanted side-effects caused by undue concentration (Anon: British Society for Antimicrobial Chemotherapy Working Party: Laboratory monitoring of antifungal chemotherapy. The Lancet. Vol. 337. pp. 1577-1580. 1991).

Paticularly in the treatment of tuberculosis, the regular intake of drugs is of great importance. Indeed, to reach the goal of elimination of tuberculosis as a public health problem, it is important to provide control programmes with an efficient tool to follow up the treatment. This can be accomplished by methods developed to detect even minor amounts of the drug or its metabolites in the body fluids. Such tests should also be carried out in chemotherapeutic studies for assessment of the efficacy of new drugs or regimens, particularly if the drugs are not taken under direct supervision.

Although rifampicin, isoniazid, pyrazinamide and ethambutol are the most commonly used drugs for the treatment

of tuberculosis, rifampicin and isoniazid are considered the first-line choice antituberculosis agent. The rifampicins are antibiotics produced by the bacterium *Streptomyces mediterranei* and is an amphoteric substance which is soluble both in organic solvents and in acid pH water. Rifampicin is metabolized by the liver, especially during its first passage through the hepatoportal system, and its principal metabolite is 25-desacetylrifampicin. The pharmacokinetics of rifampicin varies with the age of the patient and is affected by impaired liver and kidney function. In such circumstances, therapeutic drug monitoring of rifampicin might be of value in optimizing the dose. It is excreted from the human body unaltered and as its metabolites, desacetyl-rifampicin being its principal metabolite. Most of the drug is eliminated in the bile (about 80%) and some by the kidneys.

Almost all known methods of determining rifampicin concentration in body fluids, e.g. microbiological, HPLC and colorimetric methods, need a number of prior extraction steps to separate rifampicin and its metabolites from the complex mixture. Such step plays an important role in the acuracy of the assay because it is necessary to strip off rifampicin and its metabolites which became bound to body fluid proteins. But, in these methods, the removing treatment of the interferent components demands time and results in losses of the analytes, i.e. rifampicin and particularly its metabolites which are present in very low concentrations.

According to the present invention just one extraction step is used as prior treatment before drug level detection. Aqueous zinc sulfate, an appropriate solvent and the body fluid to be analyzed are mechanically mixed, and after centrifuging, the deproteinized supernatant phase is carefully recovered to determine drug and its metabolites concentrations.

The prior separation of proteins from the body fluid to permit interferents elimination before drug level analysis is known. Accordingly, Frijus-Plessen described a deproteinizing step in an assay to determine the concentration of the anti-retroviral drugs ddI, ddC and AZT (see Frijus-Plessen N., Michaelis H.C., Foth H. e Kahl G.F. (1990). "Determination of 3'-azido-3'-deoxythymidine, 2',3'-dideoxycytidine, 3'-fluoro-3'-deoxythymidine and 2',3'-dideoxyinosine in biological samples by high-performance liquid chromatography". Chrombio. Elsevier Science Publishers B.V. Amsterdam. 534: 101-107) . The proteins contained in blood are precipitated by using a saturated ammonium sulfate solution. In fact, the salting out of proteins is a well-known and frequently used method in protein purification. Scopes, R.K. (Scopes, R.K. Protein Purification Principles and Practice. Second Edition. Springer-Verlag. New York. Chapter 3. Pp 50. 1988) mention that the most effective salts used as salting out agents are those with multiple-charged anions such as sulfate, phosphate and citrate. In addition, it is cited that the cation is

relatively less important, and even so monovalent ions should be used, with $NH_4^+ > K^+ > Na^+$ in precipitation effectiveness.

Despite these assertions we have now found that the separation of proteins from complex mixtures, such as blood or plasma, is not effective unless zinc sulfate is used. Indeed, the separation of the interferent proteins by precipitating them from the drug containing body fluid is not obtained when saturated ammonium sulfate solution is used in the deproteinizing step. Moreover, according to the invention, a relative low concentration of zinc sulfate is advantageouly used. The concentration of the zinc sulfate solution may vary from 0.1M to 5M, and preferably from 0.2M to 1.0M.

Thus, the complete method for monitoring patient compliance and drugs bioavailability of the present invention comprises the following steps: (1) mixing and shaking mechanically the body fluid with aqueous zinc sulfate solution, an appropriate solvent and, optionally an anti-oxidizing agent to precipitate proteins and strip off bound drug; (2) centrifugating the mixture to obtain the separation of phases; (3) recovering the supernatant which is used for the drug concentration measurement using an accurate technique, such as a colorimetric assay or a High-Performance Liquid Chromatography method.

The solvent used in the deproteinizing step are known and depends on the solubility properties of the drug which is

being measured. In the case of rifampicin and its metabolites, despite acetonitrile/2-propanol (1:1) is preferred, several organic solvents can be used such as benzene, toluene, dichloromethane, chloroform or its mixtures. For antimonials, itraconazole and proteinase or the reverse transcriptase inhibitors, such as 2',3'-dideoxyinosine (ddI), 2',3'-dideoxycytidine (ddC) or 3'-azido-2,3'-dideoxythymidine (AZT), polar solvents, in particular water, are used.

The anti-oxidizing agents used in the deproteinizing step are also known to those skilled in the art aiming to slow down the occurrence of oxidation reactions. Ascorbic acid may be cited as an example.

Drug concentration is measured by a suitable technique. Colorimetric and HPLC methods are preferred and well known (e.g., see McConnell, J.B., Smith, H., Davis, M., Williams, R. "Plasma rifampicin assay for an improved solvent extraction technique. Br J. Cin Pharmac. 8:506-507. 1979; Acocella, G., Nonis, A., Gialdroni-Grassi, G., Grassi, C. "Comparative bioavailability of isoniazid, rifampicin, and pyrazinamide administered in free combination and in a fixed triple formulation designed for daily use in antituberculosis chemotherapy". Am Rev Respir Dis. 138: 882-5. 1988.; Ishii, M., Agata, H. "Determination of rifampicin and its main metabolites in human plasma". J Chromatogr. 426: 412-6. 1988; Vanakoski, J., Mattila, M.J., Vainio, P., Idänpään-Heikkila,

J.J. and Törnwall. "150 mg fluconazole does not substantially increase the effects of 10 mg midazolam or the plasma midazolam concentrations in healthy subjects". Int J Clin Pharmacol The. 33(9): 518-523. 1995; (see Frijus-Plessen N., Michaelis H.C., Foth H. e Kahl G.F. (1990). "Determination of 3'-azido-3'-deoxythymidine, 2',3'-dideoxycytidine, 3'-fluoro-3'-deoxythymidine and 2',3'-dideoxyinosine in biological samples by high-performance liquid chromatography". Chrombio. Elsevier Science Publishers B.V. Amsterdam. 534: 101-107).

In the case of rifampicin and its metabolites, colorimetry is the most preferred when simplicity combined with accuracy is required. The rifampicin concentration is determined by spectrophotometric measurement of the supernatant organic phase at 340 nm. But the HPLC method may also be used, and antioxidant substances can be added to the mixture of aqueous zinc sulphate, organic solvent and the body fluid to retard oxidation. Assay conditions, in this case, are also easily found in the related art, e.g. Frijus-Plessen *et al*. Figure 1 demonstrates the reproducibility and accuracy of the method of the present invention through HPLC chromatograms.

Figures 2 and 3 show the reproducibility and accuracy of the method of the present invention illustrated by HPLC chromatograms of synthetic mixtures of rifampicin, 2',3'-dideoxycytidine (ddC), 2',3'-dideoxyinosine (ddI), 3'-azido-

2,3'-dideoxythymidine (AZT) and itraconazole with body fluids.

To perform the rifampicin level detection method of the present invention, a kit containing standard solutions of aqueous zinc sulphate, organic solvent and body fluid, e.g. plasma standards, serum standards containing a known amount of rifampicin are provided. Procedure instructions may also be supplied. A typical kit of the invention consists of a solution of aqueous $ZnSO_4$ in a concentration from 0.1M to 5M, an organic solvent selected from the group of acetonitrile/2-propanol (1:1), benzene, toluene, dichloromethane, chloroform or its mixtures and a set of mixtures of plasma with rifampicin at several concentrations to obtain the standard curve for the user conditions. Particularly preferred are aqueous ZnSO in a concentration of 0,2M to 1.0M and a mixture of $CH_3CN/CH_2CHOHCH_3$ (1:1) as the solvent.

Figures 4A and 4B and Table 1 refer to the standard curves of the colorimetric assay for mixtures of rifampicin and plasma at several concentrations ranging from 0,39 to 25 µg/ml and λ = 340 nm. These standard curves for plasma extract had correlation coefficients of 0.9999 and the mean recoveries of rifampicin were at least 98%, corroborating the efficacy of the one step prior treatment of the present invention.

The method is also suitable for pharmacokinetics studies of rifampicin and its metabolites. Figure 5 shows the

rifampicin levels in plasma of two HIV positive patients with tuberculosis following oral administration of 600 mg of rifampicin, during a period of 24 hours subsequent to drug administration. The curve corresponding to the sample of one of the patients is irregular because he is suffering from hepatic problems.

Table 1: Standard Curves of Rifampicin concentration in plasma

Concentration (µg/ml)	Absorbance							Mean	Standard Deviation	Variation Coefficient
	Curve 1	Curve 2	Curve 3	Curve 4	Curve 5	Curve 6	Curve 7			
0.39	0.013	0.012	0.014	0.012	0.009	0.008	0.011	0.01128571	0.00213809	0.00668153
0.78	0.022	0.021	0.019	0.021	0.020	0.019	0.021	0.02042857	0.00113389	5.55052723
1.56	0.042	0.040	0.042	0.046	0.040	0.043	0.043	0.04228571	0.00205866	4.86846088
3.00	0.096	0.088	0.088	0.094	0.090	0.087	0.097	0.09142857	0.00415761	4.54738507
6.25	0.189	0.180	0.191	0.191	0.178	0.181	0.185	0.18500000	0.00544671	2.9441684
12.50	0.382	0.368	0.370	0.365	0.375	0.368	0.367	0.37071429	0.00587975	1.58605901
25.00	0.770	0.748	0.768	0.724	0.775	0.746	0.732	0.75185714	0.0197689	2.62934297
Correlation Coefficient	0.99997559	0.99998962	0.99983102	0.99991144	0.99986669	0.99998607	0.99994411			

The advantages of the method of the present invention as compared with available methods described in literature are: accurate determination of the drug concentration in body fluid; faster determination of a selected drug level in a body fluid; simpler technique which is useful in smaller hospital centers and field laboratories; and lower costs permitting its use in public health systems.

The following examples are illustrative of the invention and represent preferred embodiments. Those skilled in the art may know, or be able to find using no more than routine experimentation, to employ other appropriate materials and techniques, such as the above mentioned extracting substances and measuring methods.

EXAMPLE 1

This example is to illustrate the determination of rifampicin level in plasma using a colorimetric assay.

500 µl of plasma is mixed with 200 µl of 0.5M $ZnSO_4$, 500 µl of acetonitrile:2-propanol (1:1, v/v) and ascorbic acid 0,5 mg/ml in a vortex mixer and centrifuged for 3 minutes at 3,500 rpm. The spectophotometric measurement of the supernatant organic phase permit the determination of rifampicin level in plasma.

This assay lasts 15 minutes. It is a very fast procedure as compared with other assays comprising many steps for rifampicin separation and does not require expensive equipment and specialized operating personel which are

necessary in more sophisticated techniques, such as HPLC.

EXAMPLE 2

The purpose of this example is to illustrate the determination of rifampicin level in plasma using a HPLC procedure.

To 500 µl of plasma, urine or saliva containing unknown amount of rifampicin are added 250 µl of $ZnSO_4.7H_2O$ 0,5M, 1 ml of acetonitrile:2-propanol (1:1, v/v) and 0.5 mg/ml of ascorbic acid. The mixture is mechanically shaken for 5 minutes and, then centrifuged for 10 minutes at 3,500 rpm. A 20 µl aliquot of the supernatant organic phase is injected into chromatographic column.

The chromatographic operating conditions are: the mobile phase consisting of 38% of B in A, where A = 0.1M KH_2PO_4 (10% H_2O) and B = CH_3CN, pH = 3.5. The mixture is pumped at a constant flow-rate of about 2 ml/minute under a pressure of about 40 bar at room temperature, such as 30°C; the column is a RP 18 10 µm 250 x 4.6 mm column; and the detection was carried out at 254 nm.

Calibration samples were prepared by measuring 20µl of rifampicin solution. Three to six samples containing 1.25 to 20 µg/ml of rifampicin were prepared. The calibration graphs (peak area against time) were straight lines. The complete calibration was repeated every day. Retention time for rifampicin was 4 minutes as showed in figure 1.

CLAIMS

1. Method of monitoring patient compliance and bioavailability of drugs contained in body fluids comprising the following steps:

(a) mixing and shaking mechanically the body fluid with aqueous zinc sulfate solution, an appropriate solvent and, optionally an antioxidizing agent to precipitate proteins and strip off bound drug;

(b) centrifugating the mixture to obtain the separation of phases;

(c) recovering the supernatant and proceed to drug concentration measurement.

2. Method according to claim 1 wherein the concentration of the aqueous zinc sulfate solution varies from 0.1M to 5.0M.

3. Method according to claim 3 wherein the concentration of the aqueous zinc sulfate solution varies from 0.2M to 1.0M.

4. Method according to claim 1 to 3 wherein the appropriate solvent is a polar, a nonpolar or mixtures thereof.

5. Method according to claim 4 wherein the nonpolar solvent is an organic solvent selected from the group consisting of acetonitrile/2-propanol (1:1), benzene, toluene, dichloromethane, chloroform or mixtures thereof.

6. Method according to claim 4 wherein the polar solvent is selected from the group consisting of water, alcohols or mixtures thereof.

7. Method according to claim 1 wherein ascorbic acid is the antioxidizing agent used in step (a).

8. Method according to claim 1 wherein the drug concentration measurement is carried out by using a colorimetric assay or a High-Performance Liquid Chromatography method.

9. Method according to claims 1, 3, 5 and 7 wherein the drug to be analyzed is rifampicin.

10. Method according to claim 1 wherein the drug to be analyzed is selected from the group of antimonials, itraconazole and proteinase or the reverse transcriptase inhibitors.

11. Method of monitoring patient compliance and bioavailability of rifampicin contained in body fluids comprising the following steps:

(a) mixing and shaking mechanically the body fluid with aqueous zinc sulfate solution, an organic solvent selected from the group consisting of acetonitrile/2-propanol (1:1), benzene, toluene, dichloromethane, chloroform or mixtures thereof and, optionally an antioxidizing agent to precipitate proteins and strip off bound drug;

(b) centrifugating the mixture to obtain the separation of phases;

(c) recovering the organic phase supernatant and proceed to drug concentration measurement by using a colorimetric assay or a High-Performance Liquid Chromatography method.

12. Method according to claim 11 wherein the concentration of the aqueous zinc sulfate solution varies from 0.1M to 5.0M.

13. Method according to claim 12 wherein the concentration of the aqueous zinc sulfate solution varies from 0.2M to 1.0M.

14. Method according to claims 11 and 13 wherein the solvent used in step (a) is acetonitrile/2-propanol (1:1).

15. Method according to claim 11 wherein ascorbic acid is the antioxidizing agent used in step (a).

16. Method according to claim 11 wherein the rifampicin concentration is determined through spectrophotometric measurement at 340 nm.

17. Kit for measuring rifampicin concentration in a body fluid containing the following components:

(a) a standard solution of aqueous zinc sulfate optionally having an antioxidizing agent;

(b) an organic solvent selected from the group consisting of acetonitrile/2-propanol (1:1), benzene, toluene, dichloromethane, chloroform or mixtures thereof;

(c) serum standards containing a known amount of rifampicin to prepare the standard curve for the user conditions.

18. Kit according to claim 17 wherein the concentration of the aqueous zinc sulfate solution varies from 0.1M to 5.0M.

19. Kit according to claim 18 wnerein the concentration of the aqueous zinc sulfate solution varies from 0.2M to 1.0M.

20. Kit according to claim 17 wherein ascorbic acid is the antioxidizing agent.

21. Kit according to claim 17 wnerein the organic solvent is acetonitrile/2-propanol (1:1).

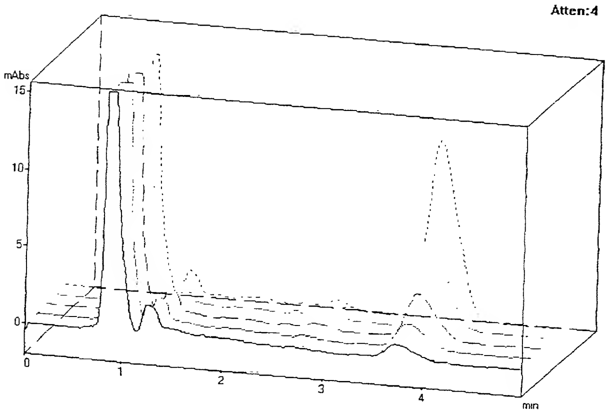

Figure 1A

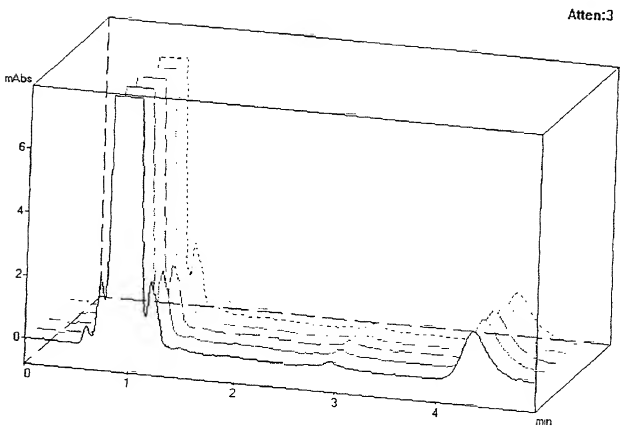

Figure 1B

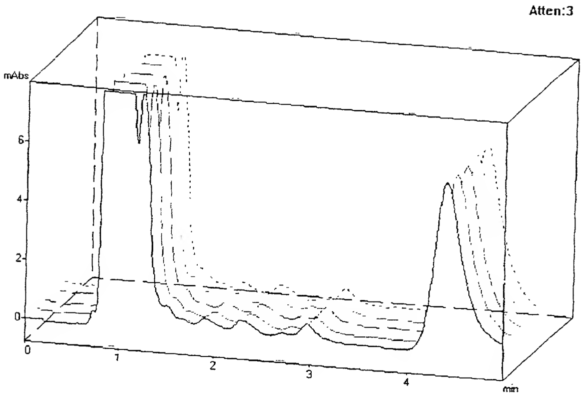

Figure 1C

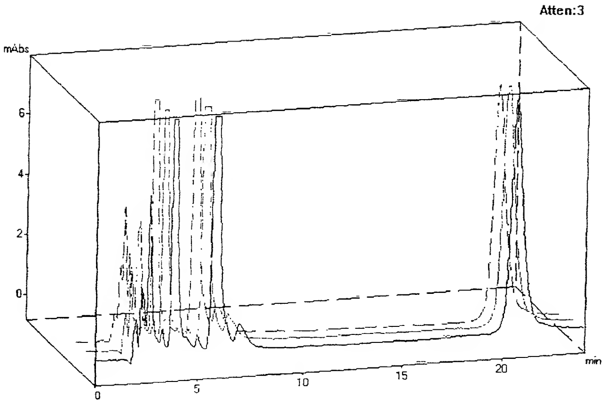

Figure 2

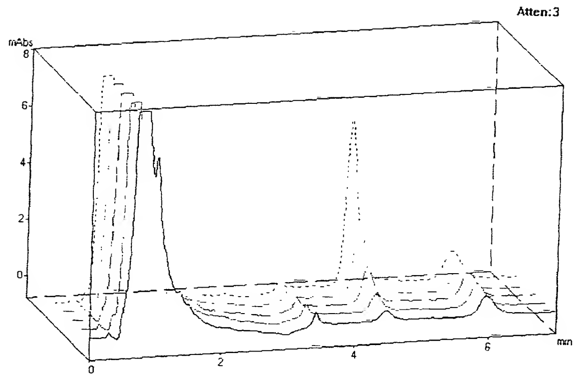

Figure 3

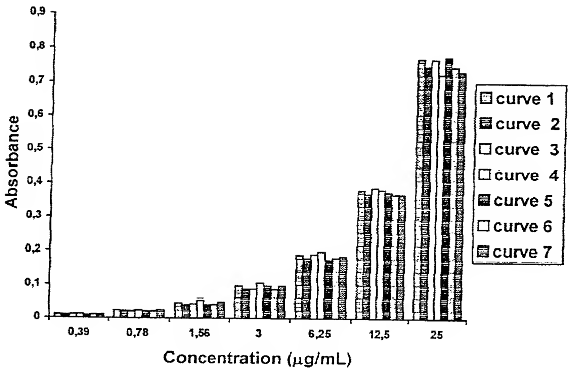
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
Absorbance
0,39
0,78
1,56
3
6,25
12,5
25
Concentration (μg/mL)
curve 1
curve 2
curve 3
curve 4
curve 5
curve 6
curve 7

Figure 4A

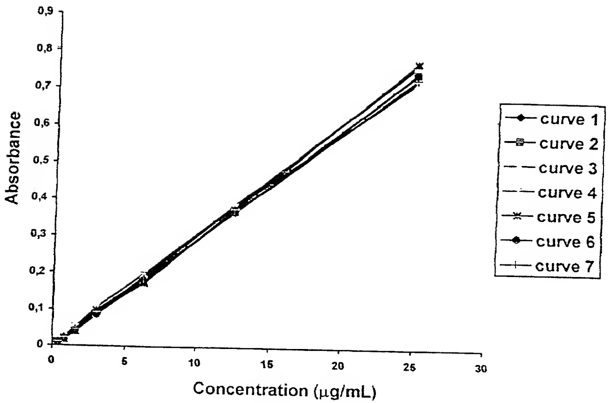
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
Absorbance
0
5
10
15
20
25
30
Concentration (µg/mL)
curve 1
curve 2
curve 3
curve 4
curve 5
curve 6
curve 7

Figure 4B

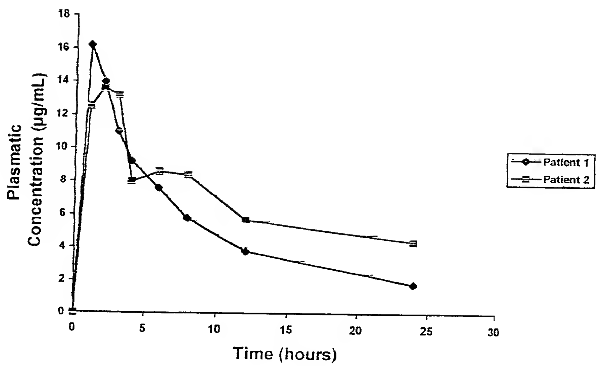
Plasmatic Concentration (µg/mL)
18
16
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30
Time (hours)
Patient 1
Patient 2

Figure 5

3673-3

#3

RULE 63 (37 C.F.R. 1.63)
INVENTORS DECLARATION FOR PATENT APPLICATION IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

MONITORING PATIENT COMPLIANCE AND BIOAVAILABILITY OF DRUGS BY DEPOTEINIZING BODY FLUIDS

the specification of which (**check** applicable **box(s)**):

☐ is attached hereto
☒ was filed on July 19, 2000 as U.S. Application Serial No. (Atty Dkt. No. 3673-3)
☒ was filed as PCT International application No. PCT/BR99/00096 on 23 November 1999
and (if applicable to U.S. or PCT application) was amended on

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Priority Foreign Application(s):

Application Number	**Country**	**Day/Month/Year Filed**
PI 9804648-9	Brazil	23 November 1998

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

Application Number	**Date/Month/Year Filed**

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s):

Application Serial No.	**Day/Month/Year Filed**	**Status: patented pending, abandoned**
PCT/BR99/00096	23 November 1999	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And on behalf of the owner(s) hereof, I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively owner's/owners' attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffry H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr. 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32955; J. Scott Davidson, ~~33489~~; Alan M. Kagen, 36178; Robert A. Molan, 29834; B. J. Sadoff, 36663; James D. Berquist, 34776; ~~Updeep S. Gill, 37334~~; Michael J. Shea, 34725; Donald L. Jackson, ~~41090~~; Michelle N. Lester, 32331; Frank P. Presta, 19828; Joseph S. Presta, 35329; Joseph A. Rhoa, 37515. I also authorize Nixon & Vanderhye to delete any attorney names/numbers no longer with the firm and to act and rely solely on instructions directly communicated from the person, assignee, attorney, firm, or other organization sending instructions to Nixon & Vanderhye on behalf of the owner(s).

29

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3-00

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